PDGF–R α Expression in Preneoplastic and Neoplastic Hepatocellular Lesions: A Rat Model N-nitrosomorpholine Stop Experiment

Su Jin Kim · Kyoung Tae Kim1
Jin Sook Jeong

Departments of Pathology and Internal Medicine, Dong-A University College of Medicine, Busan, Korea

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Corresponding Author
Jin Sook Jeong, M.D.
Department of Pathology, Dong-A University College of Medicine, 3-1 Dongdaehsin-dong, Soe-gu, Busan 602-714, Korea
Tel: 051-240-2921
Fax: 051-247-2903
E-mail: jjsjung@dau.ac.kr

Background: N-nitrosomorpholine (NNM) is a genotoxic hepatocarcinogenic agent. Preneoplastic and neoplastic hepatocyte lesions were induced in rats by oral exposure to NNM (200 mg/L) in a stop model experiment. Platelet-derived growth factor receptor (PDGF-R) is a tyrosine kinase receptor that works with PDGF, stimulating cellular growth and proliferation. The present study was designed to determine the role of PDGF-R α expression in hepatocellular neoplasms and precursors. Methods: Seventeen rats out of a starting number of 30 died. From the fifth week until the 24th week one or two rats were evaluated. Preneoplastic single cells or foci, foci of altered hepatocytes (FAH) hepatocellular adenomas (HCA) and hepatocellular carcinomas (HCC) were studied histologically, and the expressions of GSTp and PDGF-R α by immunohistochemistry. Results: At the fifth week, GSTp+ single cells showed PDGF-R α expression (20.8 ± 5.8%). At the sixth week, GSTp+ single cells, located at periportal areas, co-expressed PDGF-R α (43.4 ± 9.6%). Over the next several weeks periportal hepatocytes showed weaker PDGF-R α expression but no GSTp. GSTp+ FAH, and all HCA, demonstrated no PDGF-R α expression. However, nine out of 10 (90%) HCC showed PDGF-R α expression. Conclusions: These data showed that there were two peaks of PDGF-R α expression, and suggest that the earlier expression is related with the response to NNM-induced hepatotoxicity, and that the later response is associated to malignant transformation.

Key Words: N-nitrosomorpholine; Rat liver; PDGF receptor; Malignant transformation

N-nitrosomorpholine (NNM) is a well known compound causing hepatotoxicity and genotoxic hepatocarcinogenicity in the rat.1 Its detailed chemistry for formation of reactive intermediates and end products has not yet been clarified. Like other nitrosamines, it has been reasoned that NNM is converted by the p450 system into reactive intermediate forms that adduct to DNA bases and other biomolecules.2 Since NNM induced malignant transformation requires long and continuous exposure, NNM can be considered as both an initiator and promoter of carcinogenesis. Although the initiation and promotion of carcinogenesis may not be marked by a well defined transition, the preneoplastic lesions that develop have been well characterized.3,4 At the promotion stage, the initiated cell population is subject to clonal expansion resulting in preneoplastic lesions defined as foci of altered hepatocytes (FAH). FAH include clear, combined clear/acidophilic, acidophilic, basophilic and tigroid cell foci.3 Preneoplastic FAH are consistently found as precursors of hepatocellular adenomas (HCA) and carcinomas (HCC) that have been induced, in different species including primates, by a variety of chemical carcinogens, chronic hepadna viral infection, transgenic manipulation and hormonal imbalance.5,6 The platelet-derived growth factor (PDGF) is one of the polypeptide growth factors that signal by cell surface tyrosine kinase receptors (PDGF-R) which result in diverse cellular functions including: survival, differentiation, growth and proliferation.7-9 Several related genes have been identified that constitute a family of ligands (primarily PDGF A and B) along with their cognate receptors (PDGF-R α and β). To date, PDGF-R expression has been demonstrated in various solid tumors, such as glioblastomas and prostate carcinomas.7,8 In these solid tumors, the biological role of PDGF signaling has been suggested by autocrine stimulation of cancer cell growth and more subtle paracrine interactions that involve adjacent stroma and vasculature. In the liver, mesenchymal cells in tumor capsules and portal tracts, and hepatic stellate cells in liver fibrosis have demonstrated PDGF induced proliferation.10,11 During diethylnitrosamine induced hepatocarcinogenesis, elevated PDGF-β chains have been found in hepatocytes during the early stages, in the form of foci and nodules, suggesting malignant transformation of hepatocytes by autocrine stimulation.12 The expression of PDGF-R, in preneo-
plastic and neoplastic hepatocellular lesions, has not been described in humans and animals. Earlier hepatocyte lesions, especially FAH usually identified with glutathione S transferase placental form (GSTp), are populations that represent reactive hepatocyte injury; the mechanisms and involved factors for further selection and progression to cancer remain vague. GSTp is a well known marker for the putative preneoplastic foci in chemically induced liver injury.\textsuperscript{13,14} The present study was designed to determine the role of PDGF-R α in preneoplastic and neoplastic hepatocellular lesions which were sequentially induced during a NNM Stop Experiment in a rat model by evaluating its expression patterns using immunohistochemistry. Furthermore, we considered the probable progression sequence, resulting from consecutive lesions, in chemically induced hepatocarcinogenesis.

**MATERIALS AND METHODS**

**Experimental animals**

Thirty male Sprague-Dawley rats, weighing approximately 160 g at the start of the experiment, were purchased from Dae Han Experimental Animal Center (Daegu, Korea), were acclimatized for one week and caged in groups of three. Rats were maintained under constant conditions (22°C room temperature, 30-50% humidity and 12 h light-dark cycle) on a commercial laboratory chow, \textit{ad libitum}. All procedures were performed in accordance with the guidelines of the Experimental Animal Committee of Research Supporting Center for Medical Science, Dong-A University.

**Administration of NNM**

A potent dose of NNM (200 mg/L, Sigma Chemical CO., St. Louis, MO, USA) was used to generate rat hepatocellular carcinoma in a short period of time.\textsuperscript{15} There were thirty rats fed with water containing NNM (200 mg/L) and lab chow. NNM administration lasted for a period of seven weeks, followed by a stop experiment without NNM in the drinking water. The stop period was 17 weeks.

**Histology and classification of hepatocellular lesions**

From fifth to the 24th week, one rat at the 5th, 6th, 7th, 8th, 12th, 15th and 16th week, and two rats at the 22th, 23th and 24th week were sacrificed for investigation. Seven rats at the sixth week, six rats at the 12th week and four rats at the 14th week died. Five sagittal liver slices were obtained; they were taken from the middle lobe in the cases with no gross distinct nodular lesions, and representative sections from all HCA and HCC were investigated. Paraffin sections were stained with H&E, routinely. GSTp+ single cells and GSTp+ multicellular foci were identified with an anti-GSTp stain as shown by the immunohistochemistry. All foci consisting of at least five GSTp+ cells per cross-section were defined as FAH. FAH, HCA and HCC were classified as described previously.\textsuperscript{5} FAH were of clear, combined clear/acidophilic and acidophilic cell foci; they were glycogenic lesions, and diffusely mixed, basophilic and tigroid cell foci composed of cells with more or less reduced glycogen content. HCA were distinguished from the preneoplastic foci by the presence of a compressed surrounding liver parenchyma. The number of GSTp+ single cells, GSTp+ multicellular foci and FAH were calculated per cm\textsuperscript{2} in each liver slice microscopically and analyzed statistically; results were presented as a mean value (mean ± SD) for five liver slices in each rat. The number of HCA and HCC were counted, grossly.

**Immunohistochemistry**

The immunostaining was performed with the avidin-biotin-peroxidase complex technique, with a DAKO EnVision Kit (DakoCytomation, Glostrup, Denmark). Immunoperoxidase studies were performed on sections prepared from formalin-fixed and paraffin-embedded specimens that were dewaxed and rehydrated at graded alcohol levels. Endogenous peroxidase was blocked by dipping sections in 3% aqueous hydrogen peroxide for 10 min. The antigen retrieval was performed with 10 min microwave treatment in 10 mmol/L citrate buffer, pH 6.0. Diluted primary antibodies for anti-GSTp (1:100, DakoCytomation, Glostrup, Denmark), and anti-PDGF-R α (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, SA, USA) were treated at room temperature for 1 h. For a negative control, application of the primary antibody was omitted. After the primary antibody incubation, the sections were incubated with the secondary antibody and the avidin-biotin-peroxidase complex. The sections were lightly counterstained with hematoxylin.

The immunoreaction of GSTp and PDGF-R α located in cytoplasm of altered and neoplastic hepatocytes was evaluated. For HCA and HCC, a positive responses was defined when there was greater than 50% of the neoplastic cells immunostained.
RESULTS

General profile of hepatocellular lesions during NNM stop experiment

During the entire NNM administration period, including the initiation and promotion stages, the rats were subjected to drinking water that contained NNM for seven weeks and then drinking water without NNM for another 17 weeks. During the earlier stages of NNM induced hepatocarcinogenesis, the number of GSTp⁺ single cells and GSTp⁺ multicellular foci showed a peak at the fifth week. Over the next three consecutive weeks there were decreasing numbers of cells and by the 12th week, markedly decreased numbers (Table 1). The FAH cells that were all GSTp⁺, including glycogenic cell foci, mixed cell foci and basophilic cell foci showed a peak in number of cells at the seventh week, and then the number decreased from the eighth week. Three and two HCA were noted at the 15th and 16th week, respectively, and there were numerous regenerating cirrhotic nodules during this time. Five, two and three HCC were observed at the 22th, 23th and 24th week, respectively.

Table 1. Numbers of GSTp⁺ single cells and multicellular foci, and FAH during early stage during NNM stop experiment

<table>
<thead>
<tr>
<th>Weeks</th>
<th>GSTp⁺ single cells and multicellular foci</th>
<th>FAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>581.3 ± 54.7</td>
<td>68.8 ± 22.8</td>
</tr>
<tr>
<td>6</td>
<td>209.9 ± 47.3</td>
<td>43.2 ± 15.7</td>
</tr>
<tr>
<td>7</td>
<td>268.1 ± 56.8</td>
<td>161.5 ± 23.3</td>
</tr>
<tr>
<td>8</td>
<td>240.5 ± 29.4</td>
<td>109.5 ± 43.7</td>
</tr>
<tr>
<td>12</td>
<td>83.3 ± 9.4</td>
<td>126.2 ± 42.3</td>
</tr>
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GSTp⁺, placental glutathione-S transferase; FAH, foci of altered hepatocytes.

![Fig. 1. Expressions of GSTp (A, C) and PDGF-R  (B, D) at 6th and 8th week during NNM stop experiment. (A, B) At 6th week, GSTp⁺ periportal hepatocytes express PDGF-R  and moderate portal-periportal inflammation is present. (C, D) At 8th week, more periportal hepatocytes, expressing PDGF-R , more weakly than 6th week do not express GSTp.](image-url)
Expression of PDGF-R $\alpha$ in GSTp+ single cells and GSTp+ multicellular foci

GSTp and PDGF-R $\alpha$ were not expressed in the normal liver, including hepatocytes and bile duct epithelial cells. Strong PDGF-R $\alpha$ expression in the cytoplasm was observed in $20.8 \pm 5.8\%$ and $43.4 \pm 9.6\%$ in GSTp+ single cells and GSTp+ multicellular foci at the fifth and sixth week during the NNM stop ex-

Fig. 2. Expressions of GSTp (A) and PDGF-R $\alpha$ (B) at 6th week during NNM stop experiment. Two GSTp+ FAH (arrow heads) do not express PDGF-R $\alpha$.

Fig. 3. Expressions of GSTp (A, C) and PDGF-R $\alpha$ (B, D) of HCA at 16th week (A, B) and HCC at 22th week (C, D) during NNM stop experiment. (A, B) GSTp+ HCA shows no expression of PDGF-R $\alpha$. (C, D) GSTp+ HCC shows moderately intensive expression of PDGF-R $\alpha$. 
periment, respectively. At the sixth week, the expression of PDGF-R $\alpha$ was chiefly observed at periportal GSTp$^+$ hepatocytes (Fig. 1A, B). From the seventh week to the 12th week, the expression of PDGF-R $\alpha$ was propagated more from periportal hepatocytes into the lobular hepatocytes, although with a weak intensity; these hepatocytes were GSTp$^-$ (Fig. 1C, D).

Expression of PDGF-R $\alpha$ in FAH

All FAH cells expressed GSTp but not PDGF-R $\alpha$ (Fig. 2).

Expression of PDGF-$\alpha$ in HCA and HCC

All five HCA demonstrated a null expression of PDGF-R $\alpha$ (Fig. 3A, B). Nine out of 10 HCC (90%) showed moderately intensive expression of PDGF-R $\alpha$ (Fig. 3C, D).

**DISCUSSION**

Liver cancer is among the five leading causes of cancer death in Korea and the eighth worldwide; therapeutic possibilities are limited and prognosis is usually poor. In the present work, an attempt was made to determine the NNM associated carcinogenic process by studying PDGF-R $\alpha$ expression. Essentially, the goal was to expand on the $in vivo$ studies pioneered by Bannasch and his coworkers. Their initial and subsequent extensive investigations provided the foundation for the present approach of dose and time dependent profiles for NNM induced carcinogenesis. Thus, the goal of the present study of NNM carcinogenesis, using the expression profile of PDGFR-$\alpha$, was to expand on the molecular taxonomy of carcinogenesis.

The rat liver offers an excellent model for mechanistic studies at all stages of hepatocarcinogenesis. When evaluating GSTp as a marker, putatively initiated GSTp$^+$ single cells and GSTp$^+$ multicellular foci are detectable a few days after treatment with NNM. A portion of these cells develop into preneoplastic GSTp$^+$ FAH, some of which evolve into adenomas and carcinomas. Tumor promoters, such as phenobarbital have been shown to lead to preferential inhibition of apoptosis of preneoplastic liver cells, thereby accelerating the selective growth of the lesion. The reasons for selective growth of preneoplasia remains unclear; perhaps there is enhanced sensitivity of the preneoplastic cells to endogenous tumor promoters.

PDGF $\alpha$ receptors, that contain intrinsic tyrosine kinase activity, are dimerized and autophosphorylated on the tyrosine residues by their ligand. The phosphotyrosines on the receptor operate as high affinity binding sites for several molecules involved in the down-stream propagation of signals, resulting in diverse biological activities such as: cellular growth, survival, proliferation and differentiation. In the liver, the proliferation of hepatic stellate cells and mesenchymal cells, at portal tracts and tumor capsules, have been closely related to the PDGF-PDGFR signaling axis. Among gene expression profiles, in human HCC identified by parallel hybridization analysis of multiple protein kinase genes, PDGF receptor-$\beta$ has been reported to be preferentially expressed in tumor samples. Recently, diverse solid tumors from glioblastoma to prostate carcinoma have demonstrated expression of PDGF-R. Several reports on mutant PDGF-R $\alpha$ have shown an association with c-kit negative gastrointestinal stromal tumors. The over-expression of PDGF-R $\alpha$ may play an important role as a potential marker for metastatic colon cancer. During the cellular events of initiation, promotion and progression of hepatocarcinogenesis, participation of PDGF-R has been considered to be important but not aggressively investigated.

GSTp$^+$ single cells have been reported to have a lower DNA replication than GSTp$^-$ normal hepatocytes, and from the two cell stage onward GSTp$^+$ foci have been implicated in enhanced DNA replication. In this study, GSTp$^+$ single cells at the fifth and sixth week showed strong expression of PDGF-R $\alpha$; however, none of the GSTp$^+$ FAH showed expression. At sixth week, randomly distributed GSTp and PDGF-R $\alpha$ co-expressed single cell or multicellular foci, tended to be located at the periporal area where NNM-induced hepatocyte damage and inflammation were active. The initiated GSTp$^+$ single cell and multicellular foci were selected by apoptosis mechanisms. The PDGF-$\alpha$ expression in GSTp$^+$ single cells was thought to be related to the hepatocyte response to the toxic injury, inflammatory reaction and cell death, not with initiation or promotion of hepatocarcinogenesis; this is because a weak expression of PDGF-R $\alpha$ at the periportal hepatocytes persisted until the 12th week and those hepatocytes did not express GSTp$^+$. Even clonally expanded FAH and HCA did not express PDGF-R $\alpha$. Distinct and high PDGF-$\alpha$ expression was noted in HCC, that is, in the late stage of progression of hepatocarcinogenesis. This result is consistent with a tyrosine kinase gene expression study that showed preferential expression of PDGF-R and the fibroblast growth factor receptor in human HCC. In addition, these findings correlate well with the findings that other solid tumors showed PDGF-R $\alpha$ expression in higher graded or at a later stage of advanced metastatic tumors.
profiles, in experimental premalignant lesions and human dysplastic nodules, have not been previously reported, our results may suggest a potential approach to differentiation of premalignant tumors and HCC. For further application, advanced cellular, molecular and genetic approaches in regard to the mechanism by which PDGF-R genetic activity is turned on and overexpression occurs requires further investigation.

The results of this study demonstrated that two peaks of PDGFR-α expression occurred, and suggest that the earlier, first peak, expression was related to toxic injury of the hepatocyte, inflammatory reaction and cell death, but not with direct initiation or promotion of hepatocarcinogenesis; the later expression, the second peak, was thought to be related with NNM-induced malignant transformation.

REFERENCES